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Retinoids, analogs of Vitamin A, inhibit breast cancer cell proliferation through receptors in the super-family of nuclear transcriptional factors. 9-*cis* retinoic acid (9-*cis*-RA) is a retinoid pan agonist that activates both RAR and RXR isoforms. N-(4-hydroxyphenyl) retinamide (4-HPR) has unclear receptor selectivity, but shows promising clinical activity. No established *in vitro* model, however, has been developed to study the problem of acquired retinoid resistance for breast cancer patients.

We established an *in vitro* model by generating two stable retinoid resistant cell lines, MCF-7/LCC20[4-HPR] and MCF-7/LCC21[9-*cis*-RA]. They were generated through selection of an estrogen independent MCF-7 variant (LCC1) against increasing concentrations of 4-HPR and 9-*cis*-RA. MCF-7/LCC20[4-HPR] is stably resistant to the drug 4-HPR and shows cross-resistance to 9-*cis*-RA. However, MCF-7/LCC21[9-*cis*-RA] maintains its resistance to 9-*cis*-RA but exhibits no cross-resistance to 4-HPR. RARα, RXRβ, and RARγ RNA expression in these retinoid resistant cell lines are unaltered with respect to the parental cells, and there is a 50% reduction in the RXRα expression of LCC21[9-*cis*-RA] from the parental cell line. RARβ appears not to be present in parental or resistant cell lines. Several genes have been identified with cDNA microarrays to identify molecular pathways, and the data from the arrays should help identify genes that contribute to the acquired resistance to retinoids in breast cancer cells and should develop neural network classifiers.

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FOREWORD

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Table of Contents

INTRODUCTION:

Among women living in western societies, breast cancer is the most common malignancy with one of the highest mortality rates. Retinoids currently offer a promising alternative modality to antiestrogens. The field of retinoids, analogues of Vitamin A, has been expanding, and these compounds contribute to the growth and differentiation of many normal tissues [1]. Retinoids and antiestrogens are small hydrophobic ligands, and interact with members of the steroid/thyroid receptor superfamily. These nuclear receptors (e.g., estrogen: Estrogen Receptor [ER]; retinoids: Retinoic Acid Receptor [RAR] & Retinoid X Receptor [RXR]) are transcription factors that are activated allosterically by ligand binding turning on their respective transcriptional function. The ligand-bound receptor then forms a hetero- or homo- dimer complex to initiate the transcription of genes that may, in part, mediate the inhibition of breast cancer proliferation. As in any form of chemoprevention, acquisition of resistance to retinoids can be a problem. It is plausible that an alteration in the common pathways shared by both retinoids and antiestrogens may develop into simultaneous resistance to both classes of drugs. Coregulators are proteins or RNA that are involved in many of the nuclear receptor pathways [2]. They facilitate the promotion or repression of the transcription of hormone driven genes through the deacetylation and acetylation of histones. The exact mechanism still remains unclear. We propose to identify the coregulators and retinoid related genes that could contribute to the acquisition of retinoid resistance in breast cancer. We will use gene expression microarrays to study the expression of these co-regulators and to identify pathways and biomarkers of acquired retinoid resistance and cross-resistance in breast cancer and will also this microarray data to examine novel pattern recognition algorithms using neural networks.

PURPOSE:

We hypothesize that the loss/gain of coregulator(s) that interact with RARα is responsible for the acquisition of resistance to RAR/RXR selective retinoids in breast cancer.

TECHNICAL OBJECTIVES:

Specific Aim 1. We will select estrogen independent MCF-7/LCC1 cells against retinoids, 4HPR [unclear specificity], 9-cis-RA [pan RAR and RXR agonist], and TTNPB [RAR selective agonist]. We will evaluate the resistance and sensitivity of the newly derived and established cell lines to RAR/RXR selective retinoids and antiestrogens, TAM [ER partial agonist] and ICI 182,780 [ER antagonist], and will measure retinoid and estrogen receptor levels. We will use the cell lines to further investigate the pattern of expression of the genes identified in Aim 2.

Specific Aim 2. We will identify known coregulator(s) that interact(s) with the RARα (Retinoic Acid Receptor) using gene microarray. Then we will use a novel pattern recognition algorithm using neural networks to test the accuracy of predicting retinoid resistance phenotype. We will verify and examine their patterns of expression in the retinoid responsive cell lines MCF-7 and MDA435/LCC6 and in the established retinoid resistant cell lines, MDA-MB-231, BT-20, and MCF-7/RR [generated by selection against tamoxifen], and in the newly generated retinoid resistant ER+ cell lines from Aim 1.

BODY

At the third year, Technical Objective 1 is completed. Most of Technical Objective 2 also has been largely completed, although, the altered coregulator data has not been completed. Rather, we chose to perform gene expression microarrays to obtain a more comprehensive assessment of gene expression changes associated with acquired retinoid resistance. Retinoid generated resistant cells are necessary to study acquired retinoid resistant pathways. Stock cultures of mammary epithelial cancer cells, MCF-7, were maintained in BioFluids IMEM (Improved Minimal Essential Media) with phenol red supplemented with 5% fetal bovine serum in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. RR, MIII, LCC1, LCC20$^{4\text{-HPR}}$, and LCC21$^{9\text{-}cis\text{-RA}}$ were maintained in BioFluids IMEM without phenol red supplemented with 5% Charcoal Stripped Calf Serum.

Estrogen independent MCF-7/LCC1 was selected stepwise against 4-HPR [unknown receptor specificity] and 9-cis-RA [K_d (nM) RARα=11, β=7, γ=22; RXRα=9, β=11, γ=16]. 4-HPR is presently used in phase III clinical trials for its low toxicity and effectiveness [3;4]. Veronesi *et al.* reported that 4-HPR is effective in reducing local recurrence and contralateral breast cancer in premenopausal women with early breast cancer [5-8]. 9-cis-RA is a potent pan agonist for the RAR and RXR. These two drugs, 4-HPR and 9-cis-RA, were ideal choices to create cell line models of acquired retinoid resistance for human breast cancer due to their potential use in the clinic. The two generated cell lines were named LCC20$^{4\text{-HPR}}$ and LCC21$^{9\text{-}cis\text{-RA}}$. LCC20$^{4\text{-HPR}}$ was selected stepwise against 4-HPR up to a concentration of 15 μM, and then the drug was removed from the cells. LCC21$^{9\text{-}cis\text{-RA}}$ was selected stepwise against 9-cis-RA up to a concentration of 10 μM and 9-cis-RA was removed from the cells. After removal of the drug from the two different variants, the cells were allowed to grow without the presence of the drug for 30-60 passages. LCC21$^{9\text{-}cis\text{-RA}}$ was confirmed to be resistant to 9-cis-RA, but showed no sign of cross-resistance to 4-HPR with respect to its parental cell line MCF-7/LCC1. However, LCC20$^{4\text{-HPR}}$ is resistant to 4-HPR and exhibits cross-resistant to 9-cis-RA with respect to its parental cell line LCC1. The cross-resistance data suggests that the pathway(s) used in acquiring resistance to 4HPR may overlap the pathway(s) used in acquiring resistance to 9-cis-RA. Currently, the LCC1 cell line is being selected against other clinically useful retinoids TTNPB [K_d (nM) RARα=20, β=39, γ=51; RXRα=8113, β=4093, γ=2566] and LG1069 [K_d (nM) RARα>5000, β>5000, γ>5000; RXRα=27, β=44, γ=44]. These selected cells are now frozen down in 50% DMSO/50% of 5% CCS IMEM at −79 °C. The concentration that these cells have reached is 100 nM retinoid drug; the selection process will continue for future aims. Under treatment of TTNPB, cells' morphology is altered. They appear enlarged and flattened as the cells adapt to their treated environment.

Measurements of retinoid receptor expression profiles on LCC20$^{4\text{-HPR}}$ and LCC21$^{9\text{-}cis\text{-RA}}$ are currently in progress. In the first year, we measured the RARα and RXRα expression in the retinoid resistant cell lines. We found that there was no change in RARα expression among retinoid resistant cells, and there was a significant reduction in RXRα when LCC1 cells acquire 9-cis-RA resistance. Receptor profiles were measured with RNase Protection Assays using radiolabeled RNA probes. RNase Protection Assays of the retinoid resistant cell lines with RARγ and RXRβ riboprobes have thus been completed. During the second year, the RARγ levels appear unaltered in the retinoid resistant cell lines, LCC20^{4HPR} and LCC21$^{9\text{-}cis\text{-RA}}$ (n=3). Densitometeric analyses confirm that there are no significant changes in RARγ expression among the parental and resistant cell lines. The loss of retinoid receptor RARγ expression does not appear to be responsible the acquired resistance to retinoids. In last third year, we examined the expression of RXRβ and found no changes in expression between the parental LCC1 and its resistant variants, indicating no significant role of RXRβ in the acquired resistance to retinoids. Further analysis of the expression levels of other retinoid receptors will be completed. So far, the expression of RARβ is not present in the MCF-7 resistant variants, but further experiments and controls need to be done to verify this lost expression; this lost expression is expected because it has been reported that RARβ is lost during mammary carcinogenesis and may in fact be responsible for the malignant transformation of breast cancer [9-12]. At least three independent experiments were performed. For the RPA, all cells were fed and stripped with 5% CCS IMEM. Stripping MCF-7 was done as follows; for three

2

days, MCF-7 was washed with 5% CCS IMEM three times on the first day of stripping and washed stepwise down for the following two days. On the fourth day, RNA was extracted from the cells. RNA was obtained using Trizol Reagent (Life Technologies, Inc., Grand Island, NY) according to manufacturer's instructions. The RXRβ probes were kindly provided by Dr. Marco Gottardis and Dr. William Lamph from Ligand Pharmaceuticals. The RXRβ probes were made by subcloning 129 bp from RXRβ cDNA into Promega's PGEM vector, and the 36B4 loading control was obtained similarly from 220 bp of the 36B4 cDNA. Riboprobes were labeled with $[\alpha\text{-}^{32}P]UTP$. RPAs were performed as follows. Briefly, RNA (30 µg), 36B4 probe, and retinoid probes were hybridized overnight at 50 °C followed by digestion with RNase A. Protected fragments were separated by gel electrophoresis on a 6% acrylamide Tris-Borate-EDTA/UREA gel (NOVEX, San Diego, CA). The gel was then vacuum dried at 80 °C for 1 hr. The data was quantified by phosphoimaging screens and visualized with autoradiography.

To further characterize the cells, we are currently using cDNA microarrays on LCC20$^{4\text{-HPR}}$ and LCC21$^{9\text{-}cis\text{-RA}}$. We are using Clontech's Human Atlas Arrays that contains human cancer related genes to explore differential expression of genes between the parental cell line LCC1 and its retinoid resistant derivatives. Furthermore, we also looked upon other retinoid responsive mammary carcinoma cell lines such as MCF-7, LCC6, LCC2, and LCC9 under different treatment and medium conditions (Figure 1A). So far, we have done multiple repetitions (n=5) on each of the retinoid resistant cell lines using Clontech's Atlas Array. Each probe was generated from an independent cell culture, each culture being grown on different days using identical culture conditions. mRNA was isolated from proliferating subconfluent monolayers of each cell line. mRNA quality was determined by standard spectroscopic and gel electrophoresis analysis. Probes for the Clontech Atlas gene microarrays (Clontech, Palo Alto, CA) were prepared as described by the manufacturer [13]. Clontech's Atlas Arrays were then prehybridized, hybridized with labeled cDNA, and washed as described by the manufacturer. Atlas Array was sealed in plastic and signals detected by phosphorimage analysis.

Background signal was estimated by selecting a random, preselected area of the filter, where there is no target cDNA. The value was subtracted from all "specific" signals, producing the background-corrected estimates. The data were then normalized to account for differences in probe specific activity, hybridization, and other variables among replicates. The data from these arrays should help to identify genes that may contribute to the acquired resistance to retinoids in breast cancer cells.

Statistical analyses (t-tests) were done on each of the 597 genes on the Array comparing the parental cell line LCC1 and the retinoid resistant cell line. Genes of interest with $p < 0.05$ and fold differences ≥3 were chosen

TABLE 1: Eleven genes are selected from 597 genes based on the criteria with fold ≥3 and $p < 0.05$ between parental MCF-7/LCC1 cells and retinoid cross-resistant MCF-7/LCC20$^{4\text{-HPR}}$ cells.

GeneName	LCC1/LCC20
cyclin G1	11
interleukin-3 (IL-3) precursor	6
DNA mismatch repair protein (hmlh1)	6
neurofibromatosis protein type I (NF1)	5
MAPKK 6	4
protein serine/threonine kinase (stk2)	3
extracellular signal-regulated kinase 2 (erk-2)	3
growth factor receptor tyrosine kinase (STK-1);	3
DNA-binding protein (APRF)	3
mitochondrial transcription factor 1	3
helix-loop-helix protein (Id-2)	0.3

TABLE 2: Eleven genes are selected from 597 genes based on the criteria with fold ≥3 and $p < 0.05$ between parental MCF-7/LCC1 cells and 9-cis-RA resistant MCF-7/LCC21$^{9\text{-}cis\text{-RA}}$ cells.

GeneName	LCC1/LCC21
N-ras	8
cyclin G1	7
interferon regulatory factor 1	5
breast cancer susceptibility (BRCA2)	4
integrin beta6	3
tumor necrosis factor	3
bullous pemphigoid antigen	3
DNA mismatch repair protein hmlh1	3
protein-tyrosine kinase (JAK1)	3
extracellular signal-regulated kinase 2	3
adenosine receptor	3

and listed in Tables 1 and 2. At least eleven genes were found between the parental cell line LCC1 and the retinoid resistant cell lines.

TABLE 3: Six genes are selected from 597 genes based on the criteria with fold ≥3 and p < 0.05 between parental MCF-7/LCC20$^{4\text{-HPR}}$ cells and MCF-7/LCC21$^{9\text{-}cis\text{-RA}}$ cells.

GeneName	LCC21/LCC20
bullous pemphigoid antigen	0.34
stem cell factor (c-kit ligand)	3
RANTES pro-inflammatory cytokine	3
transcription elongation factor (SII)	4
interleukin-3 (IL-3) precursor	7
hepatocyte growth factor activator precursor	12

In the comparison between the two retinoid resistant cells, only six genes are detected with the following criteria listed in Table 3. A fewer number of genes were detected in comparison to the number of significant differences found between the parental LCC1 and the retinoid resistant cell lines. This result is not surprising since the phenotypes of the two cell lines are very similar, and the only difference between the two breast cancer cell lines is that LCC20$^{4\text{-HPR}}$ is resistant to 4-HPR and also cross-resistant to 9-*cis*-RA. The differences in gene expression seen between the two retinoid resistant cell lines could contribute to the acquired resistance to 4-HPR.

Clustering analysis is a major topic in categorizing cell lines' molecular gene expression profiles. A visual representation of a multi-dimensional space containing 597 genes is difficult, but reducing the multi-dimensional space into a 3-D space simplifies and allows us to see the clustering of each cell lines' molecular profile using a global principal component visualization analysis [14;15].

ρ:LCC1	(n=6)
:LCC9	(n=5)
:LCC21	(n=5)
:LCC20	(n=5)
:LCC6 [-ICI, FBS]	(n=3)
σ:LCC6 [+100nM ICI, FBS]	(n=3)
ω:MCF-7[CCS]	(n=1)
ϖ:MCF-7[FBS]	(n=1)
:LCC2	(n=1)

| ρ :MCF-7 derivatives | (n=24) |
| :LCC6 ± 100 nM ICI | (n=6) |

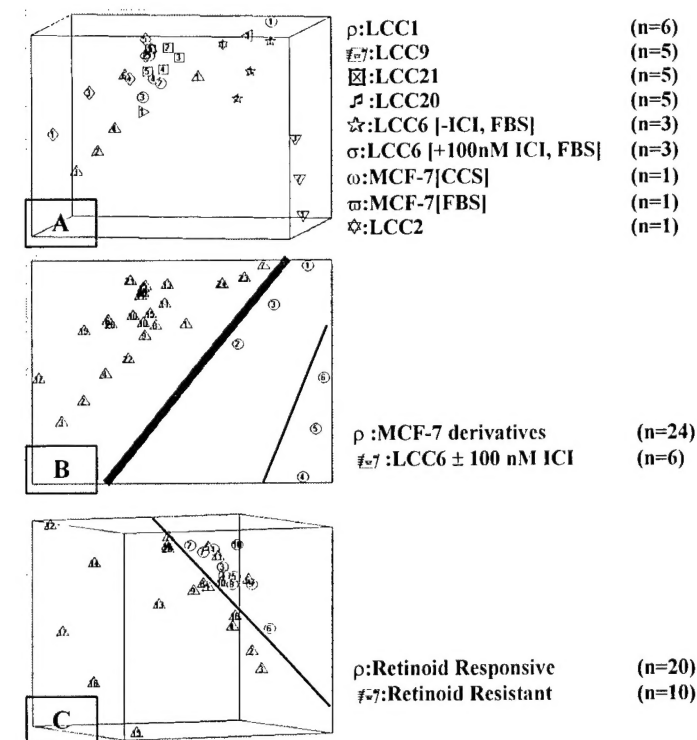| ρ:Retinoid Responsive | (n=20) |
| :Retinoid Resistant | (n=10) |

FIGURE 1: Principal component analysis projection of 30 samples from 597-D to 3-D with separation by A) nine classes based on cell types and treatments, B) two classes based on different cell origins, and C) two classes based on retinoid response

In Figure 1B, based on all 597 genes on the Clontech's Atlas Array, the clustering of the all cell lines is linear in separating the cell line origins using only the top two principal components, but the clustering of all 30 microarray experiments is non-linearly separated in terms of resistance phenotypes (Figure 1C). The top two principal components cover 80% of the total variance, whereas adding the third principal component accumulates the coverage of the total variance to 85%. So the reconstruction error in the reduction of the all gene dimensions to 3-D or 2-D is within an acceptable range.

Among the 597 genes, there are genes that are not detectable or show very little difference among the cell lines. These genes may obscure the 3-D visual representation. To reduce this obstruction, we chose specific genes based on a two-tailed t-test to see if the cell lines cluster better. Principal component visualization analysis can also be done on the specified genes, and they were all linearly separated in the 3-D space (reported in thesis dissertation). We must emphasize that the detected differential gene expression between the two cell lines must be verified by other methods such as Northern Blot Analysis and RNase Protection Assays.

The most common use of classification is the use of dendrograms formed from the Pearson's Correlation matrix [16-18]. However, this simple and common approach misclassified some of the cell models (misclassified their retinoid responsiveness). The main problem with the use of dendrograms is that the classification is strictly based on their mathematical matrix. In other words, increasing the number of replicates does not reduce the

class prediction error. We have used a novel approach by using a neural network algorithm developed by Wang et al. [14;19]. The use of a neural network approach to assess the possible relevance of genes has significant advantages over simply completing multiple PCR or RNase protection assays to confirm differential gene expression. For the neural network to classify accurately, the selected genes must be differentially expressed both to appropriate levels and in a consistent pattern in test profiles. While genes used in an accurate neural network do not have to be functionally important, the consistency of their levels and patterns of expression increases the likelihood that some of these genes are of direct biological relevance. In this regard, a predictive neural network can assist in identifying genes that may contribute to retinoid resistance.

With this novel approach, neural networks can learn as the number of replicates increase; consequently, misclassification can decrease as the number of samples increase. The use of a multilayer perceptron (MLP) neural networks allows a set of gene's levels of expressions in both the retinoid resistant and responsive cell line as the input to predict the phenotype of a cell line's response to retinoids. Each of the neural network outputs corresponds to a resistant phenotype outcome indicator; the target for that output is 1 if the phenotype outcome belongs to the retinoid resistant group and 0 if it does not.

By that process, the network output comes to approximate the posterior Bayesian probabilities of a cell line belonging to an outcome group given their gene's expression profiles [19-21]. The learning process involves updating network architecture and connection weights so that the predictive model can efficiently perform a specific classification task. The ability of MLPs to learn complex, multidimensional, and nonlinear mapping from a collection of examples makes them ideal classifiers for the predictive model. Specifically, the hidden layer and associated input/output weights are learned, through supervised training, to extract automatically the best matched features for follow-up pattern classification. This is one of the key advantages of neural predictive models over conventional regression models [20-22].

We applied the novel neural network algorithm to classify the gene expressions from breast cancer cell lines. Our two test cases consist of retinoid responsive/retinoid resistant and MCF-7 derivatives/LCC6. For retinoid responsive/retinoid resistant, the data sets have 20 data points of retinoid responsive and 10 data points of retnoid resistant. For MCF-7 derivatives/LCC6, the data sets have 24 data points for MCF-7 derivatives and 24 data points for LCC6. The average missed classifiers are shown below in Table 4.

TABLE 4: Neural Network Classification based on th 30 Different Microarray Experiments

	ret_resp/ret_rest	MCF-7/LCC6
	Missed Classifier (%)	Missed Classfier (%)
top3 genes	26.67%	3.33%
top8 genes	16.67%	0%
top20 genes	10.00%	0%

For Technical Objective 2 and future studies, we have in our possession Origene's SmartArray. In this particular microarray chip, the genes are specifically selected to be functionally related and are involved in the same regulatory pathway(s). We will use the Origene's SmartArray chip to address technical objective 2 because they have an exhaustive list of nuclear hormone receptors. These are known to differentially regulate gene expression depending upon which partner protein they bind with to form heterodimers. It also contains a list of transcriptional co-regulators (COR) which upon binding to the Nuclear Hormone Receptor (NHR) heterodimer and can function either to activate (co-activators) or inhibit (co-repressors) gene expression in response to ligand recognition. Third, the array contains an expanding group of secondary co-regulators (SCR) which, in concert with the NHR-co-regulator complex, act to either acteylate or deacteylate the chromatin and, ultimately, to either up-regulate or down-regulate expression of specific genes.

Searching for novel co-regulators using the yeast two-hybrid system has not been placed on the priority list due to technical difficulties, so we took the alternative approach by examining the levels of known coregulators. To confirm results from the SmartArray, we obtained a fragment from each of the known coregulators (e.g., SRC-1, SMRT, and CBP) through PCR [Polymerase Chain Reaction] and inserted the PCR fragments into riboprobe

vectors that will be used in RNase protection assays to study known coregulator level changes in the parental and acquired retinoid resistant cells. The assays will be performed for future studies. We have not, however, terminated the yeast two-hybrid project because the technique offers the possibility of discovering novel coregulators.

The objectives in Aim 1 are essentially complete. But we will use the cDNA microarray data and novel neural network algorithms to classify sample of unknown phenotype and to discover retinoid resistance pathways for future aims.

Key Research Accomplishments

- Established Acquired Retinoid Resistant Cell lines LCC20$^{\text{4-HPR}}$ and LCC21$^{\text{9-}cis\text{-RA}}$

- Anchorage Dependent Growth Assays of LCC20$^{\text{4-HPR}}$ and LCC21$^{\text{9-}cis\text{-RA}}$ with 9-*cis*-RA and 4-HPR

- RNase Protection Assays of retinoid Resistant cell lines and parental with RARα, RARγ, and RXRα, RXRβ.

- Constructed SMRT, CBP, and SRC-1 PCR fragments into riboprobe vectors.

- Microarray analysis on LCC1, LCC20$^{\text{4-HPR}}$ and LCC21$^{\text{9-}cis\text{-RA}}$, LCC2, LCC9, MCF-7, LCC6. Tested a novel neural network algorithm

Reportable Outcomes

Abstracts Presented at

- AACR Proceedings: The Steroid Receptor Superfamily: Celebrating the 10$^{\text{th}}$ Anniversary of the AACR Special Conference in Cancer Research: January 8-12, 1999. Renaissance Esmeralda Resort. Indian Wells, CA.

- AACR Proceedings:90$^{\text{th}}$ Annual Meeting: April 10-14, 1999. Volume 40. March 1999. Philadelphia, PA.

- Proceedings of the 1999 AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics. November 1999. Washington, DC

- AACR Proceedings: 91$^{\text{st}}$ Annual Meeting: April 1-5, 2000. Volume 41. March 2000. San Francisco, CA.'

- Society of Experimental Biology and Medicine DC Chapter: April 12, 2001. Georgetown University Medical Center. Research Building Auditorium.

Publications

Lee, R.Y., Baumann, K.H., Gottardis, M. M., Skaar, T.C., and Clarke, R. Retinoid regulation of genes and growth in E2 independent breast cancer AACR Proceedings: 88th Annual Meeting: April 12-16, 1999. Volume 38: Abstract #3043, p. 455. March 1997. San Diego, CA.

Richard Y. Lee, Todd C. Skaar, Fabio Leonessa, and Robert Clarke. Retinoid cross-resistance to 9-*cis*-RA and 4-HPR is not associated with the loss of RARα RNA expression. AACR Proceedings: The Steroid Receptor Superfamily: Celebrating the 10th Anniversary of the AACR Special Conference in Cancer Research: January 8-12, 1999. Renaissance Esmeralda Resort. Indian Wells, CA.

Lee, R.Y., Skaar, T.C., Leonessa, F., and Clarke, R. The acquisition of retinoid resistance to 4-HPR and 9-*cis*-RA in estrogen independent breast cancer. AACR Proceedings: 90th Annual Meeting: April 10-14, 1999. Volume 40: Abstract #406, p. 61. March 1999. Philadelphia, PA.

Lee, Richard Y., Skaar, Todd C., Gu, Zhiping, Leonessa, Fabio, and Clarke, Robert. Retinoid cross-resistance to 9-*cis*-RA and 4-HPR is not associated with the loss of RARα and RXRα RNA expression. Proceedings of the 1999 AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics. Published as a Supplement to Clinical Cancer Research, Volume 5: Abstract #242, November 1999. ISSN 1078-0432 Washington, DC.

Lee, R.Y., Skaar, T.C., Gu, Z., Leonessa, F., and Clarke, R. Acquiring Resistance to 9-*cis*-RA and 4-HPR in Breast Cancer is not associated with RARα and RXRα RNA expression. AACR Proceedings: 91st Annual Meeting: April 1-5, 2000. Volume 41: Abstract #3924, p. 617. April 2000. San Francisco, CA.

Wang, Y., Lu, J., Lee, R. & Clarke, R. "Iterative normalization of cDNA microarray data ." *IEEE Trans Info Tech Biomed,* in press.

Clarke, R., Skaar, T.C., Bouker, K.B, Davis, N., **Lee, Y.R.,** Welch, J.N. & Clarke, R. "Molecular and pharmacological aspects of antiestrogen resistance." *J Steroid Biochem Mol Biol,* 76: 71-84, 2001.

James, M.R., Skaar, T.C., **Lee, R.Y.,** MacPherson, A., Zwiebel, J.A., Ahluwalia, B.S., Ampy, F. & Clarke, R. "Constitutive expression of the steroid sulfatase gene in estrogen-dependent MCF-7 human breast cancer cells." *Endocrinology,* 142:1497-1505, 2001.

Gu, Z., **Lee, R.Y.,** Skaar, T.C., Bouker, K.B., Welch, J.N., Lu, J., Liu, A., Davis, N., Leonessa, F., Brünner, N., Wang, Y. & Clarke, R. "Molecular profiles of antiestrogen resistance implicate NFκB, cAMP response element binding, nucleophosmin and interferon regulatory factor-1." submitted.

Ellis, M., Davis, N., Coop, A., Liu, M., Schumaker, L., **Lee, R.Y.,** Srikanchana, R., Russell, C., Singh, B., Miller, W.R., Stearns, V., Pennanen, M., Tsangaris, T., Gallagher, A., Liu, A., Zwart, A., Hayes, D.F., Lippman, M.E., Wang, Y. & Clarke, R. "Development and validation of a method for using breast core needle biopsies for gene expression microarray analyses." prepared for submission.

Other manuscripts in preparation on the data presented in this report.

CONCLUSIONS:

We established an *in vitro* model by generating two stable retinoid resistant cell lines, MCF-7/LCC20^{4HPR} and MCF-7/LCC21$^{9\text{-}cis\text{-}RA}$. They were generated through selection of an estrogen independent MCF-7 variant (LCC1) against increasing concentrations of 4-HPR and 9-*cis*-RA. MCF-7/LCC20$^{4\text{-}HPR}$ is stably resistant to the drug 4-HPR and shows cross-resistance to 9-*cis*-RA. However, MCF-7/LCC21$^{9\text{-}cis\text{-}RA}$ maintains its resistance to 9-*cis*-RA but exhibits no cross-resistance to 4-HPR. RARα, RXRβ, and RARγ RNA expression in these retinoid resistant cell lines are unaltered with respect to the parental cells, and there is a 50% reduction in the RXRα expression of LCC21^{9CIS} from the parental cell line. RARβ appears not to be present in the parental or resistant cell lines. The data from the gene expression microarrays should help identify genes that contribute to the acquired resistance to retinoids in breast cancer cells and should develop neural network classifiers. Several genes have already been identified with cDNA microarrays to identify molecular pathways as indicated in Tables 1-3. The neural network classifiers used to evaluate our data set improve the classification of the model cell lines versus the traditional regression models. Future changes to the project would be to test an unknown sample and predict its phenotype, using the neural networks. This research is the stepping stone for future understanding of retinoid resistance applied in the clinic by providing prognostic factors, resistance mechanisms, and phenotype prediction.

Note on additional aspects of training (provided by mentor).
Mr. Lee's project took an interesting turn when we decided to use gene expression microarrays to provide a more broad assessment of molecular changes in the retinoid resistant cells. Rather than use the simple and descriptive approaches widely used in the literature, Rich became closely involved with our informatics group and engineering and computer science colleagues at the Catholic University of America. Thus, he has acquired an unique training in informatics, specifically in the visualization of high dimensional data and the construction and testing of predictive neural networks. This should be apparent from his write-up (above). These skills are in short supply and widely sought after, making it likely that Rich will have no difficulty in obtaining a high profile postdoctoral fellowship.

As a consequence of this work, Rich is very close to defining several novel signal transduction pathways that contribute to resistance to 9-*cis*-RA and to 4-HPR. Rich also has continued to do wet laboratory research, attend seminars and give presentations in our department. His thesis committee have given him permission to submit his thesis, and he will defend his thesis research At the end of October, 2001. His success and unique training would not have been possible without the generous support of this funding mechanism.